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Accurate cut-offs for predicting endoscopic activity and mucosal healing in Crohn's disease with fecal calprotectin

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ABSTRACT

Background: Fecal biomarkers, especially fecal calprotectin, are useful for predicting endoscopic activity in Crohn's disease; however, the cut-off point remains unclear. The aim of this paper was to analyze whether faecal calprotectin and M2 pyruvate kinase are good tools for generating highly accurate scores for the prediction of the state of endoscopic activity and mucosal healing.

Methods: The simple endoscopic score for Crohn's disease and the Crohn's disease activity index was calculated for 71 patients diagnosed with Crohn's. Fecal calprotectin and M2-PK were measured by the enzyme-linked immunosorbent assay test.

Results: A fecal calprotectin cut-off concentration of ≥ 170 µg/g (sensitivity 77.6%, specificity 95.5% and likelihood ratio +17.06) predicts a high probability of endoscopic activity, and a fecal calprotectin cut-off of ≤ 71 µg/g (sensitivity 95.9%, specificity 52.3% and likelihood ratio -0.08) predicts a high probability of mucosal healing. Three clinical groups were identified according to the data obtained: endoscopic activity (calprotectin ≥ 170), mucosal healing (calprotectin ≤ 71) and uncertainty (71 > calprotectin < 170), with significant differences in endoscopic values ($F = 26.407$, $p < 0.01$). Clinical activity or remission modified the probabilities of presenting endoscopic activity (100% *vs* 89%) or mucosal healing (75% *vs* 87%) in the diagnostic scores generated. M2-PK was insufficiently accurate to determine scores.

Conclusions: The highly accurate scores for fecal calprotectin provide a useful tool for interpreting the probabilities of presenting endoscopic activity or mucosal healing, and are valuable in the specific clinical context.

Key words: Fecal calprotectin. Predicting cut-offs. Endoscopic activity. Mucosal healing.

INTRODUCTION

The persistent inflammatory activity in Crohn's disease (CD) does not produce characteristic symptoms and is associated with poor prognosis (1). The assessment of symptoms has not proved to be useful in accurately establishing endoscopic activity despite the availability of instruments such as the Crohn's disease activity index (CDAI) (2). Since the main aim of treatment is to achieve mucosal healing (3), we need to base clinical criteria on tests that provide us with accurate and objective data to show the resolution or persistence of inflammatory activity in CD (4).

Ileo-colonoscopy with a biopsy is considered to be the "gold standard" for assessing intestinal inflammation, yet it is costly, not widely available and can cause complications (4-6). Although a series of specific endoscopic indices have been developed, such as the Crohn's Disease Endoscopic Index of Severity (CDEIS) and the Simple Endoscopic Score for Crohn's Disease (SES-CD), which can classify degrees of activity and correlate to a considerable degree (7), they are difficult to calculate and interpret in daily medical practice, although the SES-CD is simpler and easier to use (8). Likewise, the cut-off point is still not clearly defined for mucosal healing although some recent studies have opted for SES-CD ≤ 2 (9,10). If we also consider that colonoscopy for monitoring inflammatory activity can be complicated, then it is appropriate to identify and develop non-invasive biomarkers by producing evidence that shows that fecal biomarkers, in particular fecal calprotectin (FC), are a more accurate tool for predicting inflammatory activity in CD than blood tests (4). Such biomarkers include M2 pyruvate kinase (M2-PK), an enzyme that is expressed by continually replicating cells, such as intestinal mucus cells. This enzyme can be detected in feces by the enzyme-linked immunosorbent assay (ELISA) (11). Various studies have confirmed that M2-PK is useful for diagnosing inflammatory bowel disease (IBD) as opposed to irritable bowel syndrome (IBS), although not as accurately as FC. M2-PK levels also correlate well with the extent of activity of the disease (12), although its specificity is lower than that of FC for predicting remission (13).

FC is a calcium and zinc fixative protein found in the cytoplasm of granulocytes that represents 60% of its cytosolic proteins (14). Its presence in feces is directly proportional

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to the migration of neutrophils to the intestinal tract (15). It is simple and inexpensive to measure using ELISA, and shows a strong direct correlation between FC values and CD activity as measured by clinical, endoscopic and even histological indices (5,16). It is highly accurate for establishing endoscopic activity and is considered to be the most accurate indirect parameter for mucosal healing (17,18). It is also useful for identifying patients in deep remission who have fewer probabilities of relapsing in the short term (19).

Along these lines, Lin et al. (20) published a meta-analysis on FC accuracy in order to detect endoscopic activity, and Mosli et al. (4) have recently published a meta-analysis on the accuracy of C-reactive protein, FC and fecal lactoferrin to detect endoscopic activity in IBD patients. Both studies observed considerable heterogeneity between the various tests due to a lack of diagnostic methodology standardization as there are so few studies evaluating endoscopic activity. The two studies proposed different cut-offs for FC: 50 µg/g (5,21), 100 µg/g (17,22,23), 250 µg/g (24,25) and 274 µg/g (26), with no agreement on the optimum cut-off level.

Therefore, the clinical applicability of FC is limited as no consensus exists on the optimum cut-off point for establishing endoscopic activity. Obtaining statistical evidence of the various ranges of scores in order to accurately classify the state of "endoscopic activity" or "mucosal healing" would be an advance in aiding decision-making without the need to carry out additional tests. This in turn would help us to increase the efficiency of daily clinical practice, to better classify our patients and to provide a more personalized therapeutic treatment.

The aim of this study was to analyze the usefulness of FC and fecal M2-PK in the generation of highly accurate scores for predicting endoscopic activity and mucosal healing in monitored CD patients.

METHODS

Patients

Seventy-one patients diagnosed with CD were selected. They underwent an ileo-colonoscopy at the Juan Ramón Jiménez hospital in Huelva (Spain) and passed through a prospective and consecutive selection process based on the following inclusion criteria: CD diagnosed according to clinical, radiological, endoscopic and histological criteria; aged over 16, and informed consent given that was specific to this study. Exclusion criteria were: severe infection or inflammatory disease symptoms other than CD, extensive intestinal resections (total or subtotal colectomy), chronic consumption of non-steroidal anti-inflammatory medication, pregnancy, and upper gastrointestinal or extensive small bowel damage, all observed in a previous test.

Design

The patients diagnosed with CD included in the study were those who received a complete ileo-colonoscopy, which enabled us to gather data to calculate the SES-CD index. Firstly, the patients were interviewed and data was gathered to calculate the CDAI, and all patients were given three containers for collecting feces: one for FC, another for fecal M2-PK and the third one for microbiological tests (stool test, for parasites in feces and *Clostridium difficile* toxins); the samples were stored at -20 °C as a positive result in any of these microbiological tests was considered to be an exclusion factor. All the data were entered on a data gathering sheet specifically designed for the study and kept by the principal investigator.

Clinical and endoscopic activity

The CDAI was used to determine clinical activity by performing a quantitative assessment of its eight parameters. A CDAI score of < 150 was defined as remission; a CDAI of 150-220 was considered to be mild activity; between 221 and 450 was considered as moderate; and > 450, as severe (27,28). All the patients were given a full ileo-colonoscopy, and oral polyethylene glycol was used for bowel preparation. We studied the appearance of the mucosa and the presence of lesions in each colonic section and at the end of the ileum to calculate the SES-CD, which measures four parameters on a scale of 1 to 3 according to severity or extension (ulcers and their size, percentage of ulcerated surface, percentage of inflamed surface and stenosis) at five sections (rectum, left colon, transverse colon, right colon and ileum). This gives us a score, with the patients classified according to the endoscopic activity. Endoscopic remission is considered as SES-CD ≤ 2; mild activity as SES-CD 3-6; moderate activity between 7 and 15, and severe activity at SES-CD > 15 (29,30).

Determining the fecal biomarkers

All the patients submitted three containers of feces following ileo-colonoscopy, which were stored at -20 °C. These were later defrosted and the ELISA test was applied to measure FC (Calprest® Eurospital, Trieste, Italy) and M2-PK levels (Schebo® - Tumor M2-PK™ ELISA Stool, Giessen, Germany). Calprest® showed results ranging from 15.6 µg/g to 500 µg/g, but for those patients with > 500 µg/g we performed a dilution appropriate for the samples extracted, which resulted in a score of over 1,000 µg/g. Schebo® - Tumor M2-PK™ ELISA showed results ranging between 1.0 U/l and 20 U/l; samples which were > 20 U/l were diluted, as previously done with Calprest®, which resulted in scores above 40 U/l.

Statistical analysis

A descriptive study of the data recorded the mean age of the patient, gender distribution, years of disease evolution, and location and distribution by degrees of activity and treatment. CDAI showed a normal distribution, whereas the other variables did not. An ANOVA test, with its significance Fisher-Snedecor F-statistics test, was performed to evaluate homogeneity in the age variable, according to the SES-CD and CDAI groups. The non-parametric Mann-Whitney U test was used to compare the averages of the biomarker levels in the different groups of activity. An accuracy analysis was made by estimating the FC and M2-PK ROC curves with respect to the

SES-CD. We used the Youden index to calculate both biomarkers cut-off points with greater accuracy to establish endoscopic activity, as well as values for sensitivity and specificity, and for predictive scores: positive and negative, global accuracy and likelihood ratios (LR). A cut-off with a specificity of > 90% and LR+ of ≥ 10 was thought to present strong evidence to support the diagnostic hypothesis (endoscopic activity), and a cut-off point with a sensitivity of > 90% and LR- of ≤ 0.1 would offer strong enough proof to reject the hypothesis (31,32). Finally, the Fagan nomogram was calculated to determine the probability of endoscopic activity or mucosal healing after obtaining the biomarker score. In all cases a p-value of $p < 0.05$ was regarded as statistically significant. The statistical analysis was carried out using the SPSS 21.0, MedCalc 13.3.3 program and Excel 2013.

Ethical aspects

This study was approved by the ethics committee of the Hospital Juan Ramón Jiménez. All the patients who participated in the study signed a specific informed consent form.

RESULTS

The descriptive statistics of the patients is shown in table I. There were no significant differences regarding the age of the subjects in the SES-CD ($F = 2.103$, $p = 0.108$) or CDAI ($F = 1.945$, $p = 0.131$) groups.

Levels of FC and fecal M2-PK according to activity

Median FC levels were 71 µg/g for remission endoscopy, 196 µg/g for mild activity, 383 µg/g for moderate activity, and 575 µg/g for severe activity. Median fecal M2-PK levels were 2.4 U/l for remission endoscopy, 9.3 U/l for mild activity, 19.0 U/l for moderate activity and 22.2 U/l for severe activity. There were significant differences between some of the CDAI and SES-CD groups for FC ($\chi^2_{CDAI} = 25.442$, $p < 0.001$; $\chi^2_{SES\text{-}CD} = 39.817$, $p < 0.001$) and M2-PK ($\chi^2_{CDAI} = 7.940$, $p = 0.047$; $\chi^2_{SES\text{-}CD} = 24.709$, $p < 0.001$). In particular, we observed that the CDAI index only showed significant differences for M2-PK in the remission and moderate groups ($U_{M\text{-}W} = 177.5$, $p = 0.010$); and for FC in the remission and mild groups ($U_{M\text{-}W} = 124$, $p = 0.001$), and remission and moderate groups ($U_{M\text{-}W} = 83$ $p < 0.001$). With regard to the SES-CD index, the only statistically significant differences for M2-PK were found between the remission group and the remaining groups (mild $U_{M\text{-}W} = 76$, $p = 0.005$; moderate $U_{M\text{-}W} = 66.5$ $p < 0.001$; and severe $U_{M\text{-}W} = 23$, $p = 0.001$), but not between mild and moderate, and moderate and severe groups. Regarding FC, the only significant differences were found between the moderate and severe groups. Limiting the groups to remission or activity, we observed that there are differences in both biomarkers both for CDAI (calprotectin $U_{M\text{-}W} = 207$, $p < 0.001$, M2-PK $U_{M\text{-}W} = 406.5$, $p = 0.013$) and SES-CD (cal-

Table I. Patient descriptive and frequency characteristics

Number of patients	71		
Age (years); median (range)	41.5 (16-69)		
Female gender	38 (53.52%)		
Disease duration (years); median (range)	5.6 (0-41)		
Disease location			
Ileal	23 (32.4%)	SES-CD[c] (median, RIQ[d])	6 (2-12)
Colonic	17 (23.9%)	Mucosal healing (≤ 2)	22 (31%)
Ileocolonic	31 (43.7%)	Mild activity (3-6)	15 (21.1%)
Disease phenotype		Moderate activity (7-15)	26 (36.6%)
Stricturing	6 (8.5%)	Severe activity (≥ 16)	8 (11.3%)
Penetrating	2 (2.8%)	CDAI[e] (median, RIQ)	164 (76-259)
Non-str., non-p.	63 (88.7%)	Remission (< 150)	31 (43.7%)
Medication at endoscopy		Mild activity (150-220)	20 (28.2%)
5-ASAa	30 (42.2%)	Moderate activity (221-450)	19 (26.8%)
Corticosteroids	11 (15.5%)	Severe activity (> 450)	1 (1.4%)
Thiopurines	20 (28.1%)	Calprotectin (µg/g); median (RIQ[d])	196 (96-419)
Methotrexate	2 (2.8%)	M2-PK[f] (U/l); median (RIQ)	9.4 (2.5-21.8)
Anti-TNFαa	10 (14.0%)		
No medication	10 (14.0%)		

[a]Amninosalicylic acid; [b]Tumor necrosis factor; [c]Simple Endoscopic Score for Crohn's Disease; [d]Interquartile range; [e]Crohn's disease activity index; [f]M2-piruvate kinase.

protectin $U_{M-W} = 116.5$, $p < 0.001$, M2-PK $U_{M-W} = 217.5$, $p < 0.001$).

The interaction study of the localization and the endoscopic activity by the ANCOVA test showed no significant interaction effects in FC ($F = .367$, $p = .695$), but there were significant differences in the endoscopic activity ($F = 20.338$, $p < .001$). Similarly, there was no significant interaction in FC ($F = 1.089$, $p = 0.343$), only in the endoscopic activity ($F = 14.643$, $p < 0.001$).

In addition, to test the probability of activity *vs* non activity groups, binary logistic regression modelling was performed. Our results showed that we can obtain the probability of having endoscopic activity via a binary logistic equation using the levels of FC ($p = 0.007$) and CDAI ($p = 0.006$).

Accuracy of FC and fecal M2-PK to predict endoscopic activity (SES-CD > 2)

When considering the SES-CD classification, we observed that both M2-PK and FC present an area under the curve (the probability of correctly classifying a pair of individuals selected at random, as healthy and unhealthy/positive or negative) to suggest that both biomarkers have a high degree of accuracy for predicting endoscopic activity ($AUC_{FC} = 0.917$, $p < 0.001$; $AUC_{M2-PK} = 0.846$, $p < 0.001$). No significant differences between the ROC curves were found ($Z = 1.751$, $p = 0.079$).

Optimum FC scores for establishing inflammatory activity and mucosal healing

The best biomarker for differentiating the SES-CD groups is FC. And it was also the only variable that had levels of sensitivity, specificity, LR+ and LR- required to provide accurate scores for predicting mucosal healing and endoscopic activity.

The optimum FC cut-off point was calculated as 170 µg/g for predicting a high probability of endoscopic activity (sensitivity 77.6%, specificity 95.5%, LR+ 17.06, LR- 0.24 and global accuracy 83%; Youden index = 0.730).

To establish a second group with a high probability of mucosal healing, we extracted an FC cut-off of 71 µg/g (sensitivity 95.9%, specificity 52.3%, LR+ 1.99, LR- 0.08, and global accuracy 80%). Three groups were thus defined: endoscopic activity (FC ≥ 170), mucosal healing (FC ≤ 71), and uncertainty (71 > FC < 170). We observed significant differences in the endoscopic scores ($F = 26.407$, $p < 0.01$), as well as an acceptable consistency with the CDAI index ($\tau_b = 0.420$, $p < 0.01$).

The prevalence of endoscopic activity in our sample was 69% (pre-test probability). The Fagan nomogram calculation yielded a 97% (IC 95%: 85-100%) post-test probability of presenting endoscopic activity for FC ≥ 170 µg/g, and only a 34% probability (IC 95%: 24-47%) for a level of < 170 µg/g. The prevalence of mucosal healing in our sample was 31%. The post-test probability of presenting mucosal healing for a subject with FC ≤ 71 µg/g was 84% (IC 95%: 57-96%), and if the subject presented FC > 71 µg/g it was only 19% (IC 95%: 13-26%).

In the clinical activity group, the prevalence of endoscopic activity was 85%, and 48% for the clinical remission group. For those patients with FC ≥ 170 µg/g and clinical activity, the probability of having endoscopic activity was 100%; however, if the subjects presented clinical remission, the probabilities fell to 89%. Likewise, the probability of a subject presenting mucosal healing with FC ≤ 71 µg/g was 87% if the subject presented clinical remission, and the probability would drop to 75% if this fell within clinical activity (Fig. 1).

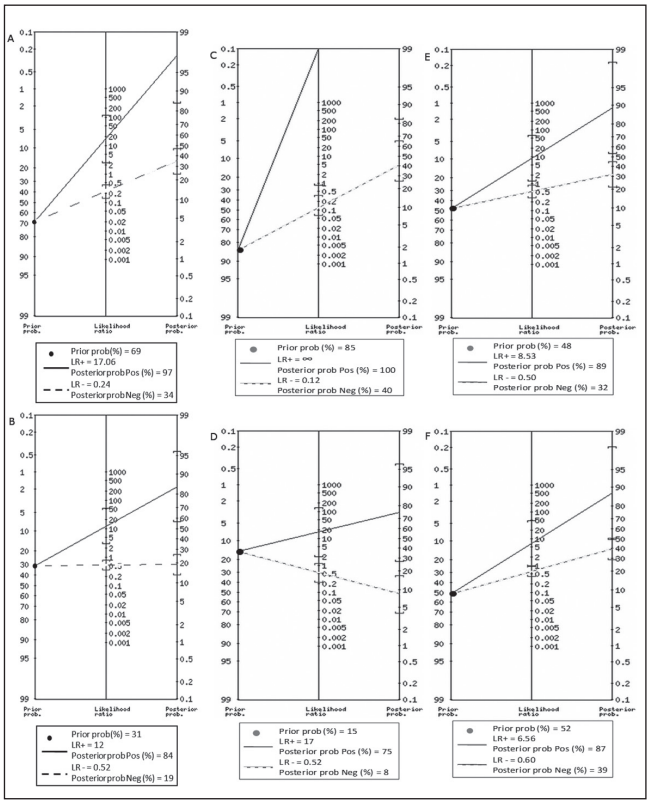

Fig. 1. Fagan plots. A. Probability of endoscopic activity for calprotectin cut-off ≥ 170 µg/g. B. Probability of mucosal healing for FC cut-off ≤ 70 µg/g. C. Probability of endoscopic activity for FC cut-off ≥ 170 µg/g patients with clinical activity. D. Probability of mucosal healing for FC cut-off ≤ 70 µg/g patients with clinical activity. E. Probability of endoscopic activity for FC cut-off ≥ 170 µg/g patients with clinical remission. F. Probability of mucosal healing for FC cut-off ≤ 70 µg/g patients with clinical remission.

DISCUSSION

FC had a diagnostic accuracy level for establishing endoscopic activity similar to that of other researchers (4).

Our optimum cut-off point of 170 µg/g reached a high level of specificity (95.5%) and a good rate of sensitivity (77.6%), similar to that achieved by Lobatón et al. (26). The Youden index for FC was higher than those recorded by Bjorkesten et al. (22) and Sipponen et al. (17), but similar to Shastri et al. (13) and Schoepfer et al. (5). Currently there is no consensus on the optimum FC cut-off point for establishing the presence of inflammatory activity or mucosal healing in patients with Crohn's disease; scores range from 50 µg/g and 272 µg/g (5,17,21-26,33).

As noted above, there are no significant differences between colonic and ilecolic in FC levels according to the endoscopic activity. It is reasonable to assume that a patient with mucosal healing must have remarkably low levels of FC, regardless of the location; similarly, patients having a larger affected area should have higher levels of FC.

The lack of consensus on fixed single cut-off limits the clinical applicability. If we lower the threshold for establishing activity or remission, we increase test sensitivity but diminish specificity, thus generating fewer false negatives but more false positives. However, if we raise the cut-off, we get fewer false positives but more false negatives; in other words, there will be lower probabilities of accurately classifying all patients with endoscopic activity.

Consequently, we calculated two cut-off points that enable us to generate highly accurate scores in order to establish mucosal healing and inflammatory activity, by producing a central low accuracy zone, or uncertainty zone, to establish the presence of endoscopic activity. We have calculated high precision scores by identifying the confirmation threshold of endoscopic activity with a specificity value of > 90% and LR+ ≥ 10, and a threshold for the exclusion of endoscopic activity at a sensitivity value of > 90% and LR- ≤ 0.1 (31,32). Real clinical usefulness is determined by the extent to which the FC enables us to step out of the area between the two thresholds (the "uncertainty zone"), which will depend on the LR value and the initial pre-test probability (prevalence).

FC presented the diagnostic accuracy levels necessary for generating highly accurate scores while M2-PK did not reach the required levels for LR+ or LR-. The most precise cut-off point for establishing endoscopic activity was FC ≥ 170 µg/g (specificity = 95.5% and LR+ = 17.24), with FC ≤ 71 µg/g (sensibility = 95.9% and LR- = 0.08) as the most accurate cut-off for establishing mucosal healing. Our sample fixed the "uncertainty zone" of FC levels between 72 µg/g and 169 µg/g. Either side of this interval, with an FC ≥ 170 µg/g the probability of finding subjects with inflammatory activity is 17 times greater than with a lower FC; and with an FC ≤ 71 µg/g the probability of finding subjects without endoscopic activity, and hence mucosal healing, is 12 times greater.

The Fagan nomogram helps us to classify the patient probabilities as percentages, and thus enables an even better clinical applicability. In our sample, the prevalence of endoscopic activity was 69%, but if a patient has an FC ≥ 170 µg/g they have a 97% probability of presenting endoscopic activity; and if they had an FC ≤ 71 µg/g, the probability would be 84% for presenting mucosal healing. In both cases, the accuracy would be sufficiently good to establish, firstly, endoscopic activity, and then, mucosal healing, which would allow us to make decisions without the need to perform a colonoscopy.

These scores are in line with the CDAI, which supports Mosli et al. (4) in their findings that FC is a useful biomarker for determining the presence of endoscopic activity, but the score should always be interpreted on an individual basis according to the specific clinical context. Endoscopic activity is more prevalent in patients with clinical symptoms than in those in clinical remission, and we should take this into account when interpreting the probabilities of presenting endoscopic activity after obtaining the FC score. In our sample, the prevalence of endoscopic activity was greater in patients with clinical activity (85%) than in those with clinical remission (48%). This implies variations in the probabilities of presenting activity with fecal FC ≥ 170 µg/g (100% *vs* 89%). Likewise, the probabilities of predicting the state of mucosal healing if FC is ≤ 71 µg/g would be 87% if presenting clinical remission, and 75% with clinical activity.

Those patients with subclinical inflammatory activity will have a worse evolution with a strong probability of a new relapse (34,35) or even complications that may require surgery (36). FC would be an ideal test to identify the presence of inflammatory activity in asymptomatic patients, enabling us to monitor the evolution of this activity and response to treatment. There is a high correlation between FC levels and the lesions in the small intestine observed by magnetic resonance enterography in patients with CD (37) or capsule endoscopy in patients suspected of EC and not yet diagnosed (38).

Combining clinical activity and FC could help us to make a series of highly accurate diagnostic and therapeutic decisions. The results and conclusions obtained may be useful in the efficient management of patients with CD in clinical practice, and used to prepare a decision diagram (Fig. 2), which incorporates the significant variables in our binary logistic regression model (CDAI and FC).

In this regard, when a patient with EC comes to our clinic, we should first check whether they are in remission or clinical activity. Then the use of the CDAI as a screening tool would be necessary. Once the subjects have been classified as in remission or activity, the values of the FC will determine how to act. Thus, we can divide the subjects into three ranges: ≤ 71 mg/g, 72-169 mg/g, and ≥ 170 mg/g, depending on whether the subjects are in "clinical activity" or "remission", which will aid us in how to proceed.

Patients who are in the area of uncertainty (72-169 mg/g) represented 28% of the total sample population. These patients, in spite of knowing their clinical activity and level of fecal calprotectin, have a low accuracy to establish the presence or activity of endoscopic mucosal healing. If these patients have symptoms they should undergo further

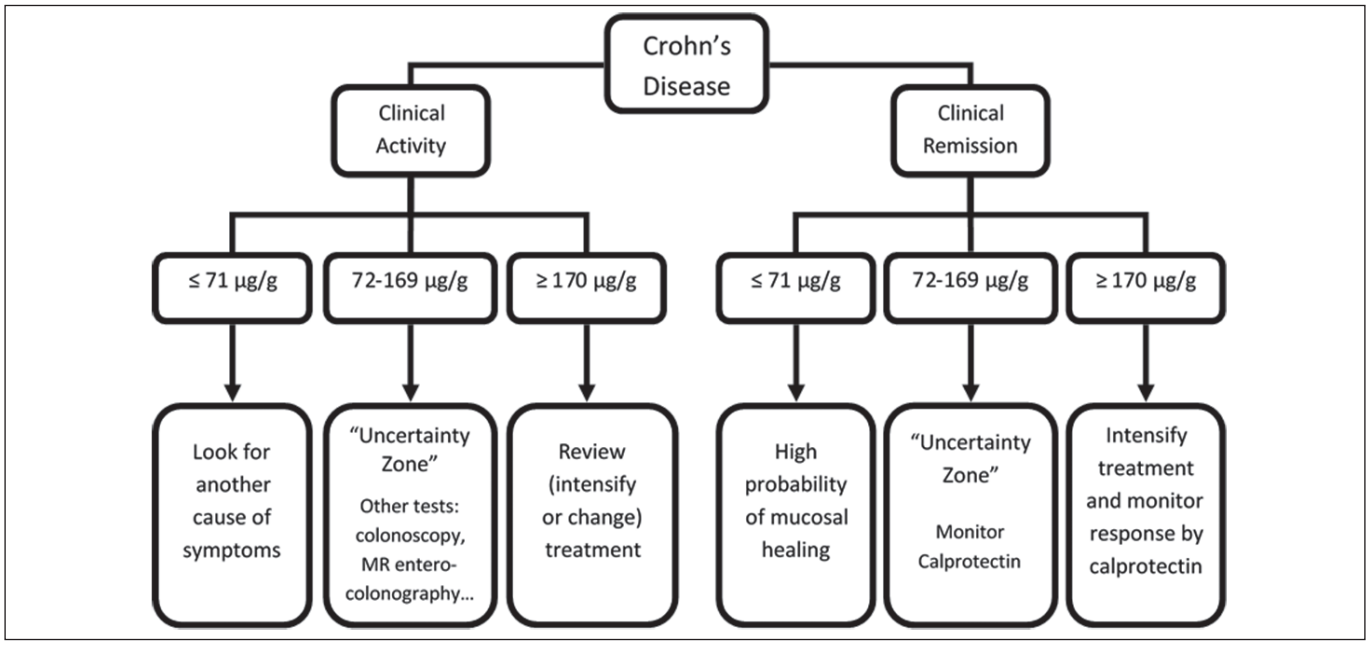

Fig. 2. Decision-making algorithm based on clinical context and FC concentration.

tests (colonoscopy and/or magnetic enteroresonance imaging) to detect presence or absence of inflammatory activity. If patients are asymptomatic, they would be candidates for monitoring fecal calprotectin in the coming months and, if the level increases above 170 mg/g, intensify treatment; otherwise, if they present symptoms, they should undergo another test to detect the presence of inflammatory activity and modify the treatment.

We recognize that our sample is highly localized, so the study would improve substantially if it included more subjects from a variety of locations. Secondly, we have analyzed inflammatory activity using only ileo-colonoscopy, hence we have taken no data for activity in segments of the small intestine other than the ileum, nor from the upper gastrointestinal tract. Finally, this is a cross-sectional study, so there is no monitoring of FC levels, which would be useful for evaluating the evolution of patients according to their activity and assessing the response to changes in treatment in a longitudinal study.

Although it is important to take into account the specific clinical context in order to interpret the probabilities of presenting endoscopic activity and mucosal healing according to the FC level, this study shows that FC is a useful tool for generating highly accurate scores for predicting the state of endoscopic activity and mucosal healing in CD patients.

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